# Are There Ethnic Differences in Deletions in the Dystrophin Gene?

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**We studied 160 cases of Duchenne muscular dystrophy (DMD) drawn from all parts of India, using multiplex PCR of 27 exons. Of these, 103 (64.4%) showed intragenic deletions. Most (69.7%) of the deletions involved exons 45–51. The phenotype of cases with deletion of single exons did not differ significantly from those with deletion of multiple exons. The distribution of deletions in studies from different countries was variable, but this was accounted for either by the small number of cases studied, or by fewer exons analyzed. It is concluded that there is likely to be no ethnic difference with respect to deletions in the DMD gene. Am. J. Med. Genet. 68:152–157, 1997** 

**KEY WORDS: Duchenne muscular dystrophy; multiplex PCR; exonal deletions; ethnic differences**

## INTRODUCTION

The incidence of Duchenne muscular dystrophy (DMD) in different countries varies from 1:2,564–1:10,309 among male births (mean, 1:4,854), while prevalence ranges from $19.5–95 \times 10^{-6}$ (mean, $65.4 \times 10^{-6}$) in the male population [Emery, 1993]. There are no studies on the incidence of DMD in India, though Roddie and Bundey [1992] reported a higher incidence (1:1,388) of the disorder among Asian Indians in Birmingham, UK, as compared to whites, Negroes, and Pakistanis (overall incidence, 1:3,761). The prevalence of DMD in India was reported to be $47.8 \times 10^{-6}$ among 20,914 males in rural Andhra Pradesh [Gouri-Devi et al., 1987]. Using the above prevalence figure and the mean worldwide incidence derived by Emery [1993], it is estimated that there are about 22,000 cases of DMD in India, while 2,687 new cases of DMD are born every year, based on recent demographic data of India [UNICEF, 1996].

The size of the DMD gene is about 2300 kb, and it has 79 exons [Bebchuk et al., 1993; Roberts et al., 1993]. The large gene size and the 5% intragenic recombination rate [Hu et al., 1988] have made diagnostic analysis complex and laborious with standard methods using DNA probes. Deletion screening with multiplex PCR offers advantages over Southern blotting, and has replaced it [Chamberlain et al., 1988, 1992].

Most mutations in the DMD gene are intragenic deletions (65%) or duplications (5%) [Koenig et al., 1988; Forrest et al., 1988; Hu et al., 1988]. A number of studies have documented the frequency and distribution of deletions in different parts of the world [Den Dunnen et al., 1989; Gillard et al., 1989; Chamberlain et al., 1992; Covone et al., 1992; Imoto et al., 1993], and some authors have suggested that these vary with ethnic origin [Sugino et al., 1989; Tsukamoto et al., 1991; Kádasi et al., 1991; Baranov et al., 1993; Ballo et al., 1994]. There have so far been only two small studies of exonal deletions among patients with DMD in India [Sinha et al., 1992; Saxena and Verma, unpublished results]. The first study involved 5 patients who were tested for two exons, while the second study was by our own group involving 70 patients who were tested for 14 exons (data not included in present study).

We set out to investigate the frequency and pattern of intragenic deletions of the dystrophin gene by multiplex PCR, and to examine whether there are any differences among Indian subjects with DMD as compared to those in other ethnic groups.

## MATERIALS AND METHODS

### Patients

We evaluated 160 unrelated male patients with DMD from all parts of India. Diagnosis was established by the characteristic clinical distribution of hypertrophy and atrophy of muscles, the markedly raised creatine kinase values, and electromyography studies in all cases. Muscle biopsy, including dystrophin staining, was performed in about 20% of cases. Age range of the patients was 3–20 years, with a mean age of 8 ± 3.06 years.

### DNA Extraction and PCR Primers

High molecular weight genomic DNA was isolated from peripheral blood leucocytes by the phenol-chloroform extraction method [Sambrook et al., 1989].

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PCR amplification of 27 exons was carried out: Pm (1), 3, 4, 6, 8, 12, 13, 17, 19, 20, 21, 22, 34, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 55, and 60. Sequences of the primers for amplification of exons Pm, 4, 8, 12, 13, 17, 19, 43, 44, 45, 48, 50, 51, and 52 were from Chamberlain et al. [1988], for exons 3, 6, 47, 49, and 60 from Beggs et al. [1990], for exons 42 and 53 from Abbs et al. [1991], for exon 22 from Bebchuk et al. [1993], and for exons 21, 34, 46, and 55 from Niemann-Seyde et al. [1992].

### PCR Conditions

Amplification was carried out in six sets of reactions (Fig. 1 a,b). PCR was performed in 25-µl volumes containing 250 ng template DNA, 12.5 pmol of each primer, 1 × *Taq* polymerase buffer (67 mmol/l Tris-HCl, pH 8.8, 16.6 mmol/l $NH_4)_2$ $SO_4$, 6.7 mmol/l $MgCl_2$, 6.7 mmol/l EDTA, pH 8.0, 0.5 mmol/l dNTPs (Boehringer-Mannheim, Mannheim, Germany), and 2.5 units of *Taq* DNA polymerase (Bangalore Genei Private Ltd., Bangalore, India). The cycling conditions were the same for all reaction tubes: 94°C for 7 min; followed by 25 cycles of 94°C for 30 sec, 52°C for 30 sec, and 65°C extension for 4 min, with a final extension of 65°C for 7 min, run in an MJ Research Thermocycler (Watertown, MA, USA). PCR products were observed on 4% ethidium bromide-stained Nu Sieve gels.

## RESULTS

Of 160 DMD patients studied, intragenic deletions were demonstrated in the dystrophin gene in 103 (64.4%) cases. Figure 1 a,b shows representative gels.

The distribution of exonal deletions is shown in Figure 2, and the percentage frequency of different exonal deletion is depicted in Table I. Altogether 117 (42.1%) of the 278 deleted exons were spread over the region involving exons 45–51, a commonly designated "hot spot" region for deletions.

Of 103 DMD patients with deletions, 62 (60.2%) subjects had deletion of a single exon and 41 (39.8%) had deletions of multiple exons. Of the 62 single exons deleted, the commonest exons involved were numbers 50 (16.1%), 45 (14.5%), 48 (14.5%), and 51 (14.5%). The commonest exons involved in multiple deletions were numbers 48 (5.3%), 49 (13.0%), 47 (11.1%), and 50 (9.7%).

The difference in phenotype according to whether the deletion involved a single exon or multiple exons was examined (Table II). The functional status of each boy was classified according to Vignos grading [Archibald and Vignos, 1959] with special reference to the abilities to run or walk, and to confinement in the wheelchair. A comparison of the distribution of subjects with deletions of single exons as compared with those with mul-

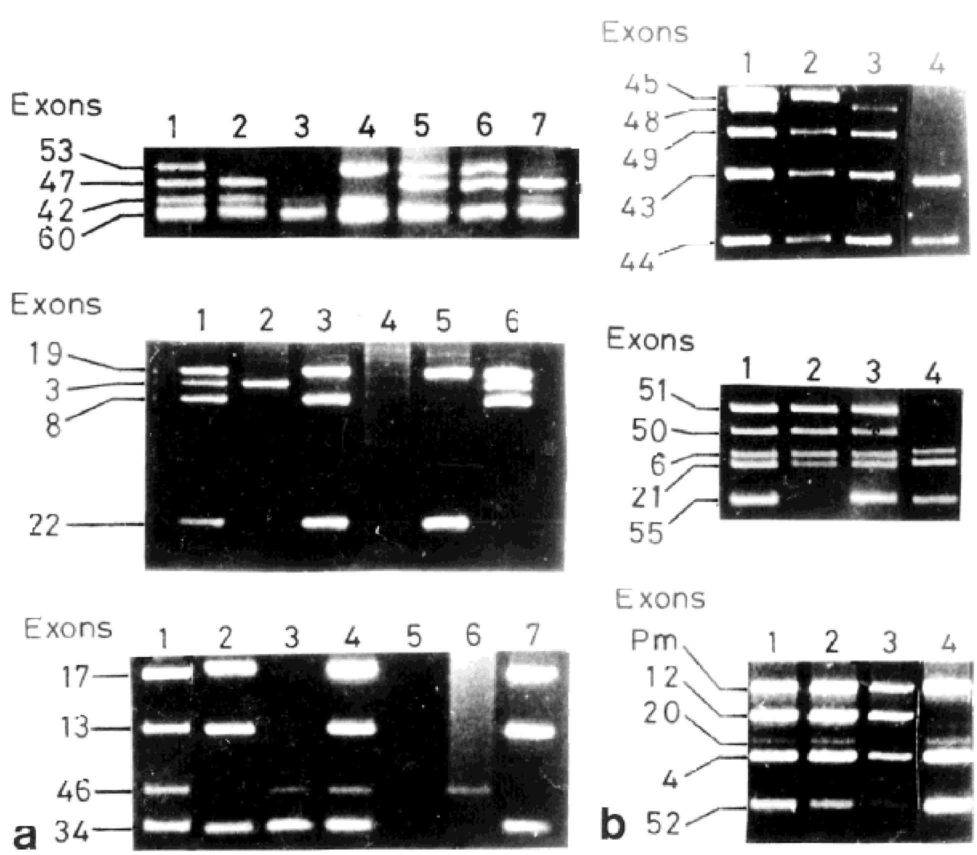


Fig. 1. **a,b:** Six different sets of reactions, amplifying 27 exons, were used for multiplex PCR analysis for DMD. In each reaction, lane 1 represents control sample from a normal male, while the other lanes are from patients. Absence of the band indicates deletion of the specific exon.

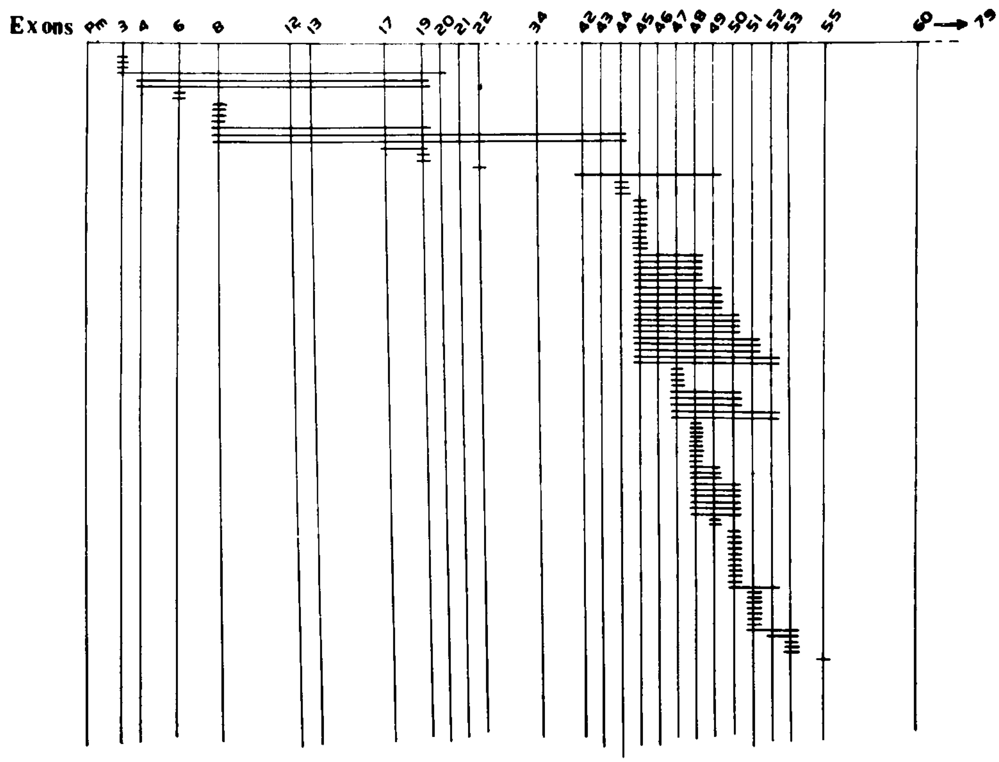


Fig. 2. Deletions of different exons as detected by PCR are displayed by one horizontal line per patient. Majority of deletions involved exons 45–51.

TABLE I. Frequency Distribution of Deletions in Different Exons

| | Number of deletions | | | |
|---|---|---|---|---|
| Exons | Single exons | Multiple exons | Total | % |
| Pm (exon 1) | | | | |
| 3 | 3 | 1 | 4 | 1.4 |
| 4 | | 3 | 3 | 1.1 |
| 6 | 2 | 3 | 5 | 1.8 |
| 8 | 4 | 6 | 10 | 3.6 |
| 12 | | 6 | 6 | 2.2 |
| 13 | | 6 | 6 | 2.2 |
| 17 | | 7 | 7 | 2.5 |
| 19 | 2 | 7 | 9 | 3.2 |
| 20 | | 2 | 2 | 0.7 |
| 21 | | 2 | 2 | 0.7 |
| 22 | 1 | 2 | 3 | 1.1 |
| 34 | | 2 | 2 | 0.7 |
| 42 | | 3 | 3 | 1.1 |
| 43 | | 3 | 3 | 1.1 |
| 44 | 3 | 3 | 6 | 2.2 |
| 45 | 9 | 19 | 28 | 10.0 |
| 46 | | 19 | 19 | 6.8 |
| 47 | 4 | 24 | 28 | 10.0 |
| 48 | 9 | 33 | 42 | 15.1 |
| 49 | 2 | 28 | 30 | 10.8 |
| 50 | 10 | 21 | 31 | 11.2 |
| 51 | 9 | 7 | 16 | 5.8 |
| 52 | | 7 | 7 | 2.5 |
| 53 | 3 | 2 | 5 | 1.8 |
| 55 | 1 | | 1 | 0.4 |
| 60 | | | | |
| Total | 62 | 216 | 278 | 100 |

tiple exons, by using the $X^2$ test, did not show any significant difference ($P = 0.37$).

We attempted to establish the beginning and the end of intragenic deletions in subjects with deletions, within the limitations of the exons tested for in the present study. Based on this information and the published sequence information on exon-intron boundaries [Koenig et al., 1989], it was determined whether the particular deletion would disturb the resultant mRNA translational reading frame. This was possible in 29 subjects with deletions. This was then correlated with clinical phenotype.

Deletions of exons 45, 45–50, and 50 are out-of-frame deletions [Koenig et al., 1989]. However, of 13 patients, 11 had a clinical phenotype of DMD, and 2 patients had phenotype of Becker muscular dystrophy (BMD). Deletions of exons 48, 45–48, 48–49, and 48–51, which are in-frame deletions, were present in 16 subjects. None of these had a phenotype of BMD, 3 had an intermediate phenotype, and 13 had a phenotype of DMD (of which 4 were wheelchair-bound). Thus, the relationship of in-frame and out-of-frame deletions to mild and severe phenotypes, respectively, was not borne out in some cases.

## DISCUSSION

The PCR technique offers advantages for the diagnosis of dystrophin gene abnormalities due to its ease of application, speed, and sensitivity. It has been routinely established in different laboratories for diagnosis of DMD, and Southern blotting with cDNA probes does not seem to be superior in terms of sensitivity [Cham-

TABLE II. Distribution of Subjects With Deletions of Single and Multiple Exons According to Their Functional Status*

| | Ability to walk | | | Wheelchair-bound |
|---|---|---|---|---|
| | Slowly | Unassisted | Assisted | |
| Single exons (n = 40)[a] | 20 (50%) | 4 (10%) | 8 (20%) | 8 (20%) |
| Multiple exons (n = 36)[a] | 14 (38.8%) | 9 (25%) | 7 (19.4%) | 6 (16.6%) |

*Functional grading of single exon deletions vs. that of multiple exon deletions ($P = 0.37$).
[a]Number of subjects on whom complete clinical data was available.

berlain et al., 1992]. In addition, the ability to multiplex-PCR many sequences simultaneously in a single tube has permitted the development of a rapid method capable of detecting about 98% of all dystrophin-gene deletions [Chamberlain et al., 1988; Beggs et al., 1990].

In the present study, using PCR amplification of 27 exons, deletions of the DMD gene were identified in 64.4% of 160 cases of DMD. Table III compares the percentage frequency of deletions in different ethnic groups in various countries.

In the case of Japanese patients, frequency of deletions [43%, Sugino et al., 1989; 43%, Tsukamoto et al., 1991] was lower than that observed in the present study and in studies in the US [Baumbach et al., 1989; Darras et al., 1988] and Europe [Hodgson et al., 1989; Gillard et al., 1989; Den Dunnen et al., 1989; Niemann-Seyde et al., 1992; Covone et al., 1992]. However, Imoto et al. [1993] reported a higher frequency (60%). Tsukamoto et al. [1991] studied only 28 patients and analyzed only nine exons. This could explain the low frequency of deletions obtained by them. Russian [Baranov et al., 1993] and Czechoslovakian [Kádasi et al., 1991] patients also showed a much lower percentage of deletions (41% and 44%, respectively) than that from other European countries [Den Dunnen et al., 1989; Niemann-Seyde et al., 1992; Covone et al., 1992]. Baranov et al. [1993] reported deletion studies in two groups of patients. In Saint Petersburg, 11 exons were examined, while in Moscow only 10 exons were analyzed. The low deletion percentage obtained is obviously due to the analysis of only a part of the gene, as not all exons recommended for standard multiplex-PCR screening were included [Chamberlain et al., 1990; Beggs et al., 1990; Abbs et al., 1991].

Soong et al. [1991] in China analyzed only 29 patients, showing deletion in 45% of them, although they used the entire cDNA probes. Likewise, Kádasi et al. [1991] studied only a few subjects (n = 32), and this may be the reason for obtaining a low percentage of deletions (44%). Ballo et al. [1994] reported deletions in 40% of subjects with DMD in South Africa. They screened 15 exons by PCR and cDNA clones spanning exons 1–19 and 44–60. Among whites (n = 32) and Indians (n = 24) they observed deletions in 50% of subjects, while in black subjects (n = 41) deletions were detected in only 24.4%. More subjects need to be examined in the different ethnic groups in South Africa before firm conclusions can be drawn. Vainzof et al. [1991] showed deletions in 40% of Brazilian patients. However, they used only two probes for Southern blotting and also studied a small number of patients (n = 47). The reason for lower frequency of deletions reported by Chamberlain et al. [1992], in a multicenter study involving 745 subjects from leading laboratories in the world, might be because only 18 primers covering nine exons were used. However, they compared the accuracy of a single PCR-multiplex amplification (nine exons) with Southern analysis using 10 cDNA probes covering the full length of the gene. The multiplex-PCR analytic method detected 82% of those deletions identified by Southern blot analysis.

From Table III it is clear that, whenever cDNA probes covering the whole gene were used for Southern analysis [Darras et al., 1988; Hodgson et al., 1989; Den Dunnen et al., 1989; Gillard et al., 1989; Baumbach et al., 1989; Imoto et al., 1993], or whenever most of the primers covering the two "hot spot" regions were used for PCR amplifications [Covone et al., 1992; Niemann-Seyde et al., 1992; present study], the frequency of deletions observed was similar. Interestingly, Covone et al. [1992] used 30 primer sets, and the frequency of deletions was almost the same as that in the present study, where 27 primer sets were used for PCR amplification.

In the present study, deletions were located mainly in one "hot spot" region (exons 45–51). Since the dystrophin gene has now been shown to have 79 exons, the area which is the "hot spot" for deletions in the case of Asian Indians is clearly the middle part of the gene. Although the percentage frequency of deletions varies in different laboratories, most deletions were observed in the middle part of the gene (exons 45–51), as in the results of the present study.

Thus, a review of the published data does not support ethnic variation in the frequency of deletions in the DMD genes, or the spread of deletions over different exons. The observed differences are explainable by a smaller number of subjects having been examined, or by analysis being restricted to a lower number of exons.

In the present study, the clinical phenotype of patients with deletion of single exons did not differ significantly from those with deletion of multiple exons. This is in conformity with earlier studies [Lindlof et al., 1988; Baumbach et al., 1989]. A better correlation exists between clinical severity of the dystrophy and the effect that an intragenic deletion has on the translational reading frame of the DMD mRNA [Monaco et al., 1988]. In the present study, we did not amplify all the exons, and Southern blotting was not

TABLE III. Comparison of Frequency of Deletions in Different Ethnic Groups*

| Authors and country | Exons tested | Deletions | Deletions (%) at exons 45–51 | Method |
|---|---|---|---|---|
| Imoto et al., 1993, Japan | Entire dystrophin cDNA | 53/88 (60%) | 47.2 blot | Southern blot |
| Tsukamoto et al., 1991, Japan | cDNA probes covering exons 4, 8, 12, 17, 19, 44, 45, 48, and 51 | 12/28 (43%) | 75.1 | PCR and Southern blot |
| Sugino et al., 1989, Japan | Entire cDNA probes | 18/42 (43%) | MCP | Southern blot |
| Yang et al., 1994, China | Exons 1, 3, 6, 8, 13, 17, 43, 47, 50, 52, and 60 | 15/41 (37%) | NM | PCR |
| Soong et al., 1991, China | Entire cDNA probes | 13/29 (45%) | 50 | Southern blot |
| Ballo et al.,1994, South Africa | Exons 3, 4, 6, 8, 13, 19, 42, 44, 45, 48, 50–53, and 60; cDNA probes spanning exons 1–19 and 44–60 | 52/128 (40%) | 65 | PCR and Southern blot |
| Hodgson et al., 1989, UK | Complete cDNA probes | 163/287 (57%) | NM | Southern blot |
| Covone et al., 1992, Italy | Brain promoter region and Pm (1), 3, 4, 6, 8, 12, 13, 16, 17, 19–22, 29, 32, 34, 41–52, and 60 | 96/152 (63%) | NM | Multiplex-PCR |
| Den Dunnen et al., 1989, Netherlands | Entire cDNA probes | 115/194 (59%) | MCP | Southern blot |
| Kádasi et al., 1991, Czechoslovakia | cDNA probes cf 56a, cf 56b, 1–2a, 2b–3, 4–5a, 5b–7, and 8 | 14/32 (44%) | NM | Southern blot |
| Niemann-Seyde et al., 1992, Germany | Pm (1), 3, 4, 6, 8, 12, 13, 17, 19, 43–52, and 60 | 35/56 (62.5%) | 57.5 | Multiplex-PCR |
| Baranov et al., 1993, Russia | Pm (1), 6, 8, 12, 17, 19, 42–45, 47, 48, 50, and 52 | 49/119 (41%) | 73.5 | PCR and Southern blot |
| Gillard et al., 1989, Canada | Entire dystrophin cDNA | 109/181 (60%) | NM | Southern blot |
| Chamberlain et al., 1992, US (multicenter study) | Exons 4, 8, 17, 19, 44, 45, 48, and 51 | 346/745 (46%) | NM | Multiplex-PCR |
| Baumbach et al., 1989, US | Entire cDNA probes | 90/160 (56%) | 69 | Southern blot |
| Darras et al., 1988, US | Entire cDNA probes | 21/32 (66%) | MCP | Southern blot |
| Vainzof et al., 1991, Brazil | cDNA probes cf 56a and cf 56b | 19/47 (40%) | NM | Immunoflorescence |
| Present study | Pm (1), 3, 4, 6, 8, 12, 13, 17, 19–22, 34, 42–44, 46–53, 55, and 60 | 103/160 (64%) | 69.7 | Multiplex-PCR |

*NM, not mentioned; MCP, mostly central part of gene.

done. Therefore, the in-frame/out-of-frame and clinical phenotype could be tested in only 29 subjects. The data did not confirm that in-frame deletions have a milder phenotype, while out-of-frame deletions have a severe phenotype. However, most of the patients were children, and the full evolution of phenotype had not taken place.

This has also been observed by other investigators [Forrest et al., 1987; Monaco et al., 1988; Lindlof et al., 1988; Baumbach et al., 1989]. These results suggest that factors other than deletion of a specific exon are important in determining the phenotype in this disease [Baumbach et al., 1989].

The rapidity and simplicity of multiplex-PCR methods, combined with knowledge of the distribution of exonal deletions in an adequate number of Indian patients, has enabled us to evolve a diagnostic strategy to help the large number of affected families for carrier screening and prenatal diagnosis.

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